(Epi)genetic characterization of chemotherapy response in ovarian cancer

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Chapter 8

Summary, Discussion and Future Perspectives
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Summary

Improving ovarian cancer patients’ outcome has been proven a difficult barrier to overcome in the last decades. Due to lack of specific symptoms, majority of patients present themselves in an advanced stage of disease. At this advanced stage, cancer cells spread all over the peritoneal cavity. Debunking surgery along with (neo)adjuvant carboplatin/paclitaxel chemotherapy is the standard treatment of such advanced stage patients. It is known that residual disease after primary surgery is a strong predictor of the survival of the patients\(^1\)-\(^2\). Furthermore, it is widely accepted that histological subtypes of ovarian cancer i.e. high-grade serous (HGSOC), low-grade serous, endometrioid, mucinous and clear cell carcinoma, originate from different pathways of tumorigenesis\(^3\). Besides different (epi)genomic alterations, these subtypes are also known for their variation in platinum sensitivity with advanced stage clear cell, mucinous and low-grade serous tumors being relatively platinum-resistant and HGSOC more platinum-sensitive. Despite good initial response rates to chemotherapy (70-80\%), most HGSOC patients will relapse with drug-resistant disease\(^4\). For HGSOC, only a few genetic driver mutations are known, such as the ubiquitous presence of TP53 mutations and BRCA1/2 mutations in a subset of patients\(^5\). In spite of a few genetic mutations, HGSOC is characterized by ample aberrant DNA methylation patterns. Therefore, the studies performed in this thesis aimed to identify novel chemoresponse related key genes by epigenomic and transcriptomic profiling with respect to chemoresponse, well-defined patient groups. In addition, we aimed to establish ovarian cancer patient-derived xenograft (PDX) mouse models as representative pre-clinical models to identify and validate (epigenomic) biomarkers.

Comparison of the DNA methylome between tumor samples from chemo-sensitive and chemo-resistant HGSOC patients, might facilitate the identification of key epigenetically-regulated, chemoresponse-related genes. In Chapter 2, we performed genome-wide next-generation sequencing with methylation-enriched genomic DNA (MethylCap-seq) of primary tumors from HGSOC patients that represent extreme responders [progression free survival (PFS) ≥ 18 months] and non-responders (PFS ≤ 6 months). After integration of expression data from the same patients, we identified FZD10 as a putative novel epigenetically-regulated gene. High FZD10 DNA methylation and low FZD10 gene expression were found in the responder patient group as compared to the non-responder group. The predictive value of FZD10 methylation and gene expression was successfully confirmed in various independent patient cohorts. Functional studies in ovarian cancer cell lines proved the epigenetic regulation of FZD10. Our results also demonstrated that FZD10 silencing sensitized cells to cisplatin. In conclusion, our findings identify FZD10 as a novel chemoresponse marker for HGSOC patients.

The DNA damage response (DDR) pathway is crucial to protect tumor cells from DNA damage-induced cell death. As part of the DDR, the Ataxia Telangiectasia Mutated (ATM) signaling axis has drawn attention as a possible new target in enhancing the cytotoxic effectiveness of radiotherapy and chemotherapy. In Chapter 3, we investigated the activation status of ATM signaling axis within the DDR by immunohistochemistry in a large, well-defined cohort of chemo-naive advanced stage HGSOC patients. All components of the ATM signaling axis showed high expression levels. In two well-defined groups with the largest contrast in treatment response, high expression of Chk2 was related to good response. We subsequently studied the effect of modulating Chk2 levels on cisplatin sensitivity of two ovarian cancer cell lines SKOV3 and A2780. Chk2 depletion abrogated the cisplatin-induced S-phase cell cycle arrest and caused increased resistance to cisplatin in long-term clonogenic survival assays. We therefore conclude that Chk2 is related to good response to platinum-based chemotherapy in advanced stage ovarian patients. These results suggest that Chk2 should not be considered a therapeutic target along with platinum-based treatment in ovarian cancer patients.
PDXs are emerging as more representative preclinical models for studying ovarian cancer than cell lines as they reflect the heterogeneity of the original tumor and preserve response to therapy. However, using PDXs for preclinical cancer research demands proper storage of tumor material to facilitate logistics and to reduce the number of animals needed. In Chapter 4, we present our panel of 45 ovarian cancer PDXs, including all major histological subtypes, with an overall take rate of 68%. Corresponding cells from mice replaced human tumor stromal and endothelial cells in second generation PDXs as demonstrated with mouse-specific vimentin and CD31 immunohistochemical staining. Furthermore, for biobanking purposes two cryopreservation methods, a fetal calf serum (FCS)-based (95%v/v) “FCS/DMSO” protocol and a low serum-based (10%v/v) “vitrification” protocol were tested. After primary cryopreservation, tumor take rates were 38% and 67% using either the vitrification or FCS/DMSO-based cryopreservation protocol, respectively. Cryopreserved tumor tissue of established PDXs achieved take rates of 67% and 94%, respectively compared to 91% using fresh PDX tumor tissue. Genotyping analysis showed that no changes in copy number alterations were introduced by any of the biobanking methods. Our results indicate that both protocols can be used for biobanking of ovarian cancer and PDX tissues. However, FCS/DMSO-based cryopreservation is more successful. Moreover, primary engraftment of fresh patient-derived tumors in mice followed by freezing tissue of successfully established PDXs is the preferred way of efficient ovarian cancer PDX biobanking.

In different studies, PDXs have been characterized for their resemblance with corresponding primary tumors at histology, at the genomic level and for treatment response. However, HGSOC PDXs have not been characterized for their global DNA methylation status in terms of proving their suitability for future epigenetic studies. In Chapter 5, we compared the DNA methylome of primary tumors from HGSOC patients with their corresponding PDXs to determine their epigenomic stability among generations using genome-wide methylation arrays. Aims of this study were a) to explore how representative HGSOC PDXs are for their corresponding primary the tumors’ methylome and b) to evaluate the effect of epigenetic therapy and cisplatin on putative epigenetically regulated genes and their related pathways. Only 0.6-1.0% of all analyzed CpGs (388,696 CpGs) changed significantly during propagation showing that HGSOC PDXs were epigenetically stable. Treatment of F3 PDXs with decitabine caused a significant reduction in methylation in 10.6% of CpG sites in comparison to untreated PDXs, whereas cisplatin treatment had a marginal effect on the PDX methylome. Pathway analysis of decitabine-treated PDX tumors revealed several putative epigenetically regulated pathways (e.g. Src family kinase pathway). Particularly, C-terminal Src kinase (CSK) gene was successfully validated for epigenetic regulation in different PDX models and ovarian cancer cell lines. Low CSK methylation and high CSK expression were both associated with better overall survival in HGSOC patients. Our results encourage the future application of PDXs for cancer epigenome studies.

In comparison to other tumor types such as breast, lung and colorectal cancers, only targeted therapies have slowly found their application in HGSOC. The success of many targeted therapies relies on a variety of factors like whether the drug reaches its target and also on the expression (level) of these specific growth factors [like vascular endothelial growth factor (VEGF) and Insulin growth factor-1 (IGF-1)] and their receptors in patient tumors. Further, these expression levels can change during chemo- and/or targeted therapy, affecting treatment efficacy. In agreement to this, both elevated VEGF and IGF-1R levels have been documented to be involved in chemoresistance in ovarian cancer\textsuperscript{6,7}. The VEGF-A antibody bevacizumab is currently part of standard care in combination with platinum-based chemotherapy\textsuperscript{7,8}. Similarly, IGF-1R-targeting antibodies, like AMG-479, and IGF-1R tyrosine kinase inhibitors such as OSI-906 have been evaluated in clinical trials, either alone or in combination with chemotherapy in ovarian cancer. Hence, development of non-invasive methods for detection of multiple tumor-related proteins (like VEGF and IGF-1R) in patients may help to select targeted therapies
and monitor their behavior during cisplatin treatment in time. In Chapter 6, we tested the feasibility of dual wavelength near-infrared fluorescence (NIRF) imaging in multiple ovarian cancer PDXs established from 10 patients, using the monoclonal antibodies bevacizumab (anti-VEGF) and MAB391 (anti-IGF-1R) coupled to the NIRF dyes IRDye-800CW and IRDye-680RD, respectively. In vivo kinetics of IRDye-800CW labeled human VEGF-A targeted tracer bevacizumab displayed a rapid tracer decline in PDX tumors 24 hrs after injection. Co-injected, IRDye-680RD labeled human IGF-1R-targeted tracer MAB391, however, resided for over 6 days in PDX tumors depending on the IGF-1R positivity. Quantification showed a large variation in maximum average radiance for both tracers, demonstrating the clinically relevant heterogeneity of tracer uptake between tumors. When compared to vehicle-treated PDXs, elevated levels of both tracers were found in cisplatin-treated PDXs. Furthermore, a rapid decline of both tracers 24 hrs after co-injection was observed in cisplatin-treated PDX tumors indicating impairment of IGF-1R-mediated tracer trapping. Our findings encourage future application of NIRF imaging in PDXs and patient tumors to monitor several targets simultaneously, during drug treatment for developing novel therapeutic strategies.

To identify key genes that modulate platinum-response, we took the advantage of publicly available HGSOC expression datasets that included clinical information of patients. In Chapter 7, we applied functional genomic mRNA (FGmRNA) profiling, a tool to filter out non-genetic from gene expression data, on a large set of HGSOC patients (n=422, all stage III-IV) to identify genes that were associated with PFS, as a surrogate marker for chemoresponse in HGSOC patients. Expression of 303 genes was significantly associated with PFS. Among higher expressed genes significantly associated with poor PFS, we found MAD1L1, PRKD1, SUPT20H, NFKBIB, MMP24-AS1 and IGF-2R. Genes associated with poor PFS were enriched in biological processes with GO terms cell cycle, chromosome, mitosis, catabolic and microtubule-related processes. Higher expressed genes like MPP24-AS1, BAG2, NEDD8, CASP2, MRSP11, EVI5, NFX1 and PARP1 were significantly associated with better PFS. GO terms linked to cell locomotion activity, transmembrane and vesicle-mediated transport, DNA damage repair and cytoplasmic processes were enriched for genes associated with better PFS. In conclusion, by applying FGmRNA-profiling, we identified genes associated with PFS (i.e. chemoresponse) and their related biological processes in a clinically well-defined subset of HGSOC patients treated with platinum-based chemotherapy.

**Discussion and future perspectives**

Development of better treatment strategies for ovarian cancer has been hampered by 1) lack of novel targets that can be used to introduce targeted drugs to increase survival or to overcome resistance to platinum containing chemotherapy and 2) a lack of robust chemoresponse markers to stratify patients into categories with different therapy responses or to select patients that qualify for other treatment regimens. Furthermore, there is a need for better pre-clinical models that recapitulate the genomic and epigenomic features of patients’ tumors and take into account the inter- and intra-tumoral heterogeneity. In this thesis, we have addressed these key issues of ovarian cancer treatment with particular focus on platinum-based chemotherapy resistance in HGSOC.

**Chemoresponse (epi)genomic markers: from bench to bedside**

In this thesis, much effort has been made to identify and validate novel (epi)genomic chemoresponse markers by both discovery-driven as well as candidate-driven research approaches (Chapter 2, 3 and 7). DNA methylation-based biomarkers are relatively more stable than expression-based biomarkers. In addition, they are functionally related to gene expression, allowing high diagnostic sensitivity and are easily detectable in body fluids presenting DNA methylation as a promising epigenetic biomarker for clinical purposes. In this thesis, robust epigenetic chemoresponse markers like FZD10 and CSK were identified, which could be putative therapeutic targets to
overcome chemoresistance (Chapter 2 and 5). Since we have determined the predictive and prognostic value of our identified chemoresponse markers in chemo-naïve primary HGSOC (Chapter 2, 3 and 5), it would be of considerable interest to investigate the effect of chemotherapy on the methylation and expression status of these markers. There are only a few studies that have examined acquired resistance by investigating longitudinal epigenetic changes pre- and post-chemotherapy using cell lines and/or patient tumors. Notably, when we compared the global methylation pattern of PDX-36 in the vehicle and cisplatin-treated mice (Chapter 5), we did not find major differences in methylation status after platinum therapy. However, this comparison was based on a PDX model established from one HGSOC patient. Therefore, more studies are required that examine the (epi) genomic status of matched tumor samples pre- and post-chemotherapy with clearly defined clinical measures of platinum-resistant disease. Although routinely performed secondary surgery is no longer considered beneficial in ovarian cancer obtaining these samples is challenging, but not impossible.

Currently, all published (epi)genome-wide studies on epigenetic chemoresponse markers are mostly correlative, showing statistical associations between DNA methylation as an epigenetic biomarker prior to treatment and clinical outcome. Therefore, we need more functional approaches to investigate the consequences of epigenetic changes on the development of cellular resistance, but also to identify possible therapeutic interventions. In this thesis efforts have been made to functionally validate several candidate chemoresponse markers for their epigenetic regulation and their role in chemoresponse (Chapter 2, 3 and 5).

We have also investigated the role of the DNA damage response pathway in platinum-based chemotherapy response either by pathway-driven approaches (ATM-axis of DDR pathway in Chapter 3) or meta-analysis on large expression dataset of HGSOC patients (Chapter 7). Besides genomic and epigenomic alterations in BRCA1/2, an additional group of low-frequency mutations (PALB2, RAD51, Fanconi-anemia pathway) was reported to have a common tumorigenic mechanism linked to homologous recombination (HR) deficiency. Together these alterations account for up to half of the advanced stage HGSOC. Recent evidence suggests that mutations in the HR pathway are highly related to better platinum-based chemotherapy responses. Furthermore, targeting HR pathway components, for instance by pharmacologic targeting of oncogenic transcription factors ETS, MYC, and E2F mediated signaling that drive BRCA expression, has been shown to enhance the cytotoxicity of conventional chemotherapy. For similar reasons, synthetic lethality i.e. exploiting the defect in one repair pathway by targeting another repair pathway, for instance PARP inhibition in HR deficient tumors, has proven its value for ovarian cancer. Therefore, inhibition of cell cycle checkpoints like Chk1 and Chk2 has also been proposed to be a therapeutic option, since these inhibitors enhance sensitivity towards DNA damaging agents like cisplatin in clear cell ovarian cancer, breast cancer, and head and neck cancer. However, in Chapter 3, we have demonstrated that Chk2 depletion induced a cisplatin-resistant phenotype in ovarian cancer. Likewise, a recent mutational analysis of HGSOC tumors showed that CHK2 mutations have detrimental effects on the nuclear localization signal and were associated with poor therapeutic response and overall survival of HGSOC patients. These results emphasize that the efficacy of cell cycle checkpoint targeting is cancer type dependent and does not universally lead towards more platinum sensitivity.

Maximizing power of models to study chemoresistance

Modeling clinically relevant mechanisms of drug resistance is challenging. PDXs might be the right model to mimic the clinical scenario of developing platinum resistance. In this thesis, substantial efforts have been made for developing ovarian cancer PDX models and characterizing them at histological, genomic as well as epigenomic level for studying chemoresponse markers and chemoresistance mechanism (Chapter 4 and 5). These ovarian cancer PDX models better reflect tumor heterogeneity, patient’s chemoresponse and development
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of chemoresistance. Notably, whole genome analysis on an autopsy case demonstrated a shift in expression-based subtype, from an immune-reactive primary tumor to a relapsed tumor with “bad prognosis” mesenchymal subtype. This observation signifies the importance of longitudinal analysis with paired sequential samples to dissect the complexity of intrinsic primary resistance and acquired secondary resistance. PDX models would certainly be of great advantage in imitating these clinical scenarios (avatar). Furthermore, generation of PDXs from tissue obtained by fine-needle aspirations or even circulating tumor cells (CTCs) (also known as CTCs-derived xenografts, CDXs) avoids the need for more primary tumor material and invasive procedures. In addition, CDX models allow generation of chemotherapy sensitive and resistant PDXs from the same patient over time, to test novel therapy regimens for overcoming chemoresistance, and to understand drug resistance mechanisms. Furthermore, due to the maintenance of the tumor heterogeneity and chemo-naivety, PDXs offer the possibility of functional imaging to monitor dynamic changes in expression of therapeutic targets during treatment and the effect of their expression on therapy response, as shown in this thesis (Chapter 6).

Although PDXs are great models to recapitulate various features of cancer, they have some drawbacks. Major concerns include the gradual loss of the human microenvironment, the lack of a fully functional immune system and clonal selection, which are all known to be of crucial importance for therapy response and resistance mechanisms. Although initially harvested PDX tumors show stromal infiltration from human origin, in later generations these stromal components are overtaken by cells of mouse origin and in some cases already present 100 days after engraftment. On the other hand, the mouse microenvironment in PDX tumors does offer a unique opportunity to study species-specific expression analysis of stroma, its contribution to prognosis and therapy response as well as to acquisitently study the human cancer epigenome without interference of human stromal components in PDX tumors (in Chapter 5). Recent insights indicate the importance of the immune system in therapeutic responses, implying that caution needs to be taken when evaluating results from these immunocompromised PDX models. However, new methods are emerging to compensate this disadvantage of PDX models. Two recent studies showed that a humanized immune system can be achieved in mice, either by co-transplanted human hematopoietic stem cells (HSCs) and tumor cells into immunodeficient mice or first transplantation of HSCs to make humanized immune mouse model (XACT-mice model) followed by implantation of the primary patient tumor. With both methodologies, it was observed that a humanized immune system was present in mice for long-term (more than one year) and tumor cells grew without evidence of rejection. Another bottleneck of PDX models is clonal selection that occurs due to the selective pressure of PDX engraftment. Propagation to different generations may induce changes in PDX tumors in comparison to the original patient tumors. Although the process of clonal selection of PDX tumors during establishment and further growth over generations remain elusive, a very recent study proposed that only a limited number of minor clones dominate breast cancer PDXs after several passages. Polyclonality, however, was still observed.

Unlike PDXs, genetically-engineered mouse models (GEMMs) have a fully functional mouse immune system and are known as good preclinical models for studying carcinogenesis and recapitulating gene specific pathogenesis. Furthermore, they have been used for investigating resistance mechanisms to conventional chemotherapy and targeted drugs, however not in ovarian cancer yet. Recently, few HGSOC GEMMs were reported developing from the fallopian tube after manipulation of genes like p53, BRCA1/2, PAX8, PTEN and DICER that highly recapitulate HGSOC pathogenesis. However, current GEMMS rely on limited oncogenic alterations, which might not be sufficient to recapitulate the complexity of HGSOC cancer genomes. Therefore, GEMMS and PDXs should be utilized in a complementary manner to evaluate the efficacy of personalized therapies and to study mechanisms of therapeutic resistance. For clinical implementation, however, the challenge is the duration of both of these processes. The co-clinical trial is an alternative to speed up the process. A proof-of-principle
study was carried out using GEMMS for KRAS-mutant lung cancer to evaluate combination therapy of MEK inhibitor selumetinib along with docetaxel-based chemotherapy. This study not only provided guidance for the outcome of treatment regimens but also demonstrated that a defect in an additional tumor suppressor (Lkb1) caused primary resistance to this combination therapy in GEMMs, which might explain inconsistent responses in patients. Similarly, another study was conducted with PDX models, where 232 treatment regimens with 63 drugs were tested in PDXs from 14 refractory advanced cancer patients. Effective regimens were identified for 12 of these patients. In addition, a high throughput in vivo screening of 62 treatments across six indications was assessed in a PDX clinical trial (PCT) using ~1,000 PDXs of various tumor types with a diverse set of driver mutations. This first PCT predicted not only clinical trial drug responses but also revealed novel therapeutic resistance mechanisms and predictive response biomarkers. However, the need for large panels of PDXs for performing such PCT makes this a ‘Herculean task’ and certainly requires collaboration among research groups and pharmaceutical companies that would enable large multi-centre investigations. In addition, the variability in take rates and the slow tumor growth rates of PDXs make these trials very challenging. In this respect, cancer organoids may offer an easier and faster method, extending the possibilities of high throughput drug screening combined with genetic characterization for patient selection. Currently, the organoid model has been reported for several cancer types such as colorectal, ductal pancreatic, and prostate cancer and demonstrated high throughput drug screening for targeted as well as for epigenetic therapies. However, being an in vitro system, they lack tumor microenvironment and hence reduce the predictive value of organoids. Recently, development of an in vitro-grown vascularized and complex tissue by combining tumor cells with endothelial cells and mesenchymal stem cells was reported, and this platform may enable us for future in vitro drug testing with tumor microenvironment. Conclusively, a combined approach of utilizing organoids for high throughput drug screening and PDX for the in vivo translation of candidate drugs would be highly recommended for finding the most optimal drug combinations for patients.

Future chemotherapy resistance management in ovarian cancer:

Figure 1 summarizes a possible approach to improve management of chemoresistance in ovarian cancer. In Stage I (pre-clinical phase), chemoresponse biomarkers will be identified using (epi)genomic profiling of patients and clinically relevant models (PDXs and primary tumor cells). Furthermore, mechanistic knowledge of chemotherapy resistance will be collected from consecutive samples of a heterogeneous patient population. This can be achieved through experimental research as partly demonstrated in this thesis using appropriate clinically relevant models like PDXs. Ideally, this includes three steps: 1) Each tumor is mirrored in a pre-clinical mouse model together with its treatment response, 2) The drivers of chemoresistance resistance are identified as targets for personalized medicine by analyzing (epi)genomic profiles, and 3) Combinational therapy regimens and schedules are optimized and evaluated in mice and/or organoids before delivering to patients. All this information will be stored as a genotype-phenotype database for future reference. In Stage II (clinical phase), validation of the response to the novel combination therapy, as derived from Stage I would be performed. Taking advantage of patient information (including genetic and clinical information), treatment response phenotypes and corresponding drug resistance mechanisms from the ‘genotype-phenotype’ database, a computational model will be generated that can stratify future cancer patients for their chemoresponse and predict the optimal personalized precision therapies. The concept of using combination therapy to prevent drug resistance is not new in cancer management. However, an additional strategy should be the inclusion of (epi)genomic data collected from patients during treatment. This might help to convert cancer into a chronic, manageable condition.
Identification of chemoresponse markers and targets for combination therapy

Stage-I

Patient modelling

(epi)genomic profiling

PDXs Primary 2D and 3D culturing

Treatment

Pathways analysis and chemoresistant mechanisms

Stage-II

Prospective validation of chemoresponse markers and combination therapies

(epi)genomic profiling

Computational modelling

Omics-phenotype database

Database

Personalized therapy

Figure 1. Future chemotherapy resistance management in OC

Conclusion

In conclusion, results presented in this thesis contribute to our knowledge regarding the complex interaction between (epi)genome and chemoresistance in ovarian cancer. Future studies are needed to exploit this knowledge further by performing functional assessment of the novel identified chemoresponse markers or therapeutic targets along with their validation in prospective patient cohorts. Together this will improve management of chemoresistance in HGSOC and ultimately survival of these patients.
References


7. Zeller, C.


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